LVI. THE PROPERTIES OF PURE GLUTATHIONE¹.

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THE sulphur-containing peptide glutathione was first isolated by Hopkins [1921] in a non-crystalline form. He showed that it contained cysteine and glutamic acid, and the analytical figures seemed to indicate that it was a dipeptide containing those two amino-acids alone. Like cysteine itself, the substance in neutral aqueous solution rapidly absorbed oxygen, becoming converted into the oxidised or disulphide form. Hopkins showed that this disulphide form was rapidly reduced back to the sulphydryl form by animal tissues, so that it seemed clear that part of the oxygen normally absorbed by the respiring tissues was being utilised by the glutathione system.

While the nature of the tissue systems which reduce the disulphide group is not yet clear, the reduction is due in part, as Hopkins and Dixon [1922] showed, to factors which are not destroyed by heating the tissue to 100°. If for instance fresh muscle was minced, thoroughly extracted with water, heated to 100° and finally extracted with alcohol, it was found that the resulting "thermostable preparation" was still able vigorously to reduce oxidised glutathione. An aqueous suspension of such a preparation was unable to absorb oxygen, but on the addition of a little disulphide-glutathione (which of course takes up no oxygen by itself) an oxygen uptake was at once established. It was shown that the reduction of the disulphide group was due to "fixed —SH groups," *i.e.* sulphydryl groups, apparently forming part of the protein structure, which were not soluble in water and which were unable to react directly with oxygen (or methylene blue). These groups became oxidised by reducing the added disulphide, and the reduced glutathione formed then reacted with the oxygen.

The work of Warburg and Sakuma [1923] showed that the apparent autoxidation of cysteine was in fact due to small traces of catalytic metals (iron or copper) present as impurities. They showed that absolutely metal-free cysteine solutions absorbed oxygen only at a negligible rate, that the addition of a trace of ferrous salt to cysteine enormously accelerated its oxidation, and that its oxygen uptake was abolished by adding a little HCN, which inactivated the metals. These observations were confirmed and extended to glutathione by Harrison [1924], who showed that haematin compounds were also catalytically

¹ A brief account of some of the main points of this work was communicated to *Nature*, Oct. 5, 1929.

active. These facts did not, however, seriously affect the position as far as the biological action of glutathione was concerned, as in any case the tissues contained the iron necessary to make the glutathione autoxidisable.

The "thermostable system" was later studied in greater detail by Hopkins [1925], who showed that the total amount of disulphide which could be reduced per gram of tissue preparation was equivalent to the fixed —SH groups therein, as also was the total amount of methylene blue reduced by the tissue in presence of a little oxidised glutathione. The amount of oxygen taken up by the system was, however, much larger, amounting to several times the equivalent of the —SH groups originally present. The extra oxygen thus taken up was in fact utilised by the system for oxidation of the proteins present. The mechanism of this protein oxidation is not yet clear, but it appeared to be linked in some way with the oxidation of the sulphydryl group of glutathione, and it was thus dependent ultimately upon the fixed —SH groups, which maintained the glutathione in its reduced form. When these became exhausted all oxygen uptake ceased. Other proteins containing —SH groups (*e.g.* denatured egg-albumin) were also oxidised by glutathione, but proteins with no —SH groups (*e.g.* natural egg-albumin) were not.

Hopkins also showed that the sulphydryl form of glutathione (though not its disulphide form) strongly catalysed the autoxidation of unsaturated fats and fatty acids, particularly in acid solution. The phenomena observed were complex [Hopkins, 1925; Allott, 1927], and the mechanism of the catalysis is still unknown. It is clear, however, that the process is of a different nature from those described above, since the oxidation was shown not to depend upon the oxidation of the reduced glutathione, and indeed to occur equally well under conditions where glutathione is not autoxidisable. The system did not reduce methylene blue.

At this time glutathione was still thought to be a dipeptide of cysteine and glutamic acid. Later, however, Hunter and Eagles [1927] threw some doubt on this structure, and suggested that serine might also be a constituent of the molecule. Johnson and Voegtlin [1927] later published an apparent confirmation of the original view of Hopkins, who, however, reinvestigated the matter, and succeeded [Hopkins, 1929] in finding an elegant method for the ready isolation of glutathione in the pure crystalline state. He then found that the substance was a tripeptide containing cysteine, glycine and glutamic acid in equivalent amounts. Shortly afterwards Kendall, McKenzie and Mason [1929], working quite independently, also succeeded in obtaining crystalline glutathione, and showing that it was a tripeptide composed of those amino-acids.

This work showed that the earlier preparations of glutathione, with which all the work on oxidation catalysis had been carried out, were in fact impure, although they undoubtedly contained a large proportion of the tripeptide. The analytical figures make it probable that they contained free cysteine in addition. In view of these facts it was necessary that previous work on the

catalysis of oxidations by glutathione should be reinvestigated with the pure crystalline material. The results of such an investigation are given in the present paper. It was soon evident that in several respects the behaviour of crystalline glutathione is very different from that of the earlier preparations, and the study of these differences has yielded results which we believe will be of importance in the elucidation of the glutathione problem.

EXPERIMENTAL.

The oxygen uptakes were measured in Barcroft manometers as modified by Dixon and Elliott (private communication). The total volume of liquid in each flask was always 3 cc. Any CO_2 formed was absorbed by KOH solution. Metal-free water and buffer solutions were used throughout, and the vessels were carefully freed from metals with acid and washed with pure water before use.

The crystalline glutathione was made from yeast by the method of Hopkins [1929]. Nitrogen and sulphur determinations corresponded excellently with the theoretical values for the tripeptide formula. Several different preparations were used, and all behaved in the same way.

The (lipin-free) thermostable tissue preparations were made by the method of Hopkins [1925].

Oxygen uptake of glutathione in aqueous solution.

The crystalline glutathione, like the older preparations, absorbs oxygen when shaken in air in phosphate buffer or water at p_{H} 7.6. The total amount of oxygen taken up corresponds with that calculated on the basis of the equation

For example, in eight consistent determinations of total oxygen uptake under these conditions the mean value found was 99 % of the theoretical. The rate of absorption of oxygen at p_{H} 7.6 and 20° is about 30 mm.³ per 5 mg. glutathione per hour. This is definitely lower than that of most of the older preparations, and varies much less from one preparation to another than was the case with them.

Its oxidation is strongly inhibited by $M/1000$ cyanide, and completely prevented by $M/100$ KCN. The oxidation is therefore presumably due to the presence of traces of catalytic metals, as in the case of cysteine.

There is, however, one respect in which crystalline glutathione differs completely in behaviour from cysteine (and from the earlier preparations of glutathione). Whereas the addition of a trace of iron or copper salt greatly accelerates the uptake of oxygen by cysteine or impure glutathione, the oxidation of crystalline glutathione is not accelerated at all by the addition of iron or copper at p_{H} 7.6, or by haematin in low concentration. Therefore the catalytic metal cannot be the limiting factor in the oxidation of the new tripeptide preparations. In Fig. 1 is shown the effect on the oxidation of old and new glutathione

preparations of adding 0.001 mg. of iron. It will be seen that, whereas the rate of oxygen uptake in the first 20 minutes has been doubled by the metal in the case of the impure glutathione, that of the crystalline preparation is quite unaffected. The addition of this amount of iron to cysteine would, according to Warburg and Sakuma's [1923] figures, produce an additional uptake of roughly 100 mm.³ O₂ per hour, which is of the same order as the effect with the impure glutathione.

The absence of any accelerating effect with pure glutathione is the more remarkable in that quite large amounts of iron can be added without producing the slightest acceleration: the oxidation still proceeds at its original slow rate under conditions where the velocity of oxygen uptake by cysteine or impure glutathione becomes relatively enormous.

Fig. 1.

Fig. 2.

Fig. 1. Effect of iron on autoxidation of impure and crystalline glutathione. A, 5 mg. old glutathione + 0.001 mg. Fe; B, 5 mg. old glutathione; C, 5 mg. crystalline glutathione + 0.001 mg. Fe; D, 5 mg. crystalline glutathione. All in 3 cc. phosphate buffer p_H 7.6 at 18°.

Fig. 2. Effect of haematin on autoxidation of impure and crystalline glutathione. A, 10 mg. crystalline glutathione; B, 10 mg. crystalline glutathione + 0.05 mg. haematin; C, 10 mg. old glutathione; D, 10 mg. old glutathione + 0.05 mg. haematin. All in 3 cc. phosphate buffer p_H 7.6 at 40°. The sample of impure glutathione was different from that used for Fig. 1.

The behaviour with haematin is also interesting. Small concentrations of haematin behave like free iron, having no effect on the velocity of oxidation of the crystalline glutathione, but accelerating that of the impure preparations, as shown in Fig. 2.

With larger amounts of haematin, however, a definite acceleration is produced (as shown later in Fig. 13), although the catalytic activity is still small compared with that observed with cysteine. It is not yet quite clear how these results are to be interpreted.

A further point of difference between the crystalline and impure glutathione is that while the latter reduces haematin at a moderate rate, pure glutathione is unable to do so. In two Thunberg tubes were placed 5 cc. phosphate buffer (p_H 7.6) containing 0.1 mg. haematin and 5 mg. pure and impure glutathione respectively. The tubes were then evacuated and allowed to stand at room temperature. After 10 minutes the tube containing the impure glutathione was opened and examined with the spectroscope after the addition of pyridine. Strong absorption bands of pyridine-haemochromogen were observed, indicating that reduction of the haematin had taken place. After 30 minutes the other tube was examined in the same way, but no trace of haemochromogen could be detected, showing that the pure glutathione had been quite unable to reduce the haematin.

It is clear from the results previously given that although the oxidation of glutathione is due to catalysis by metals (and further evidence for this is given below) the slowness of the oxidation of the crystalline preparations is not due to any lack of catalytic metals, and the rate of oxidation must be determined by some other factor. The facts seem most readily explained by the assumption that the active catalyst is not the metal itself, but a complex of the metal with some other substance present in traces in the glutathione. The free metal ions are clearly catalytically inactive in the oxidation of glutathione, and they are unable to form active complexes with the glutathione itself. (In most other iron-catalysed reactions it is generally supposed that the catalyst is an iron complex, and not free iron; for instance in the oxidation of cysteine the catalyst is a complex of iron with cysteine itself.) The oxidation then would depend on the co-operation of two factors—the metal, and the complex-forming substance. On this assumption, which was fully justified by later work, the slow rate of oxidation of the crystalline preparations is due to the very small amount of the complex-forming factor present in these preparations—even smaller than the amount of catalytic metals. This is therefore the limiting factor in this case, and the addition of further amounts of metals will have no effect on the velocity. With the impure glutathione preparations, on the other hand, where the extra substance is present in very much larger amount, the amount of metal will probably be small in comparison, and will become the limiting factor. In this case any extra metal added will be converted into the active complex, and the velocity will be increased.

In order to test this hypothesis we attempted to free glutathione from the catalytic impurity by recrystallisation. A sample of crystalline glutathione was recrystallised several times from water; and after this treatment it was found that the rate of oxygen uptake had fallen to a little over half that of the original preparation. (No oxidation of the glutathione had taken place during the recrystallisation, as the p_H was of course acid throughout, and it was carried out in evacuated vessels.) As metal-free water and vessels had been used in the recrystallisation it seemed possible at first that the diminished velocity was due to a diminution of the iron-content. The addition of iron to

the solution had, however, no effect on the velocity, as shown in Fig. 3, so that the effect was clearly not due to the removal of iron. This experiment, while affording evidence of the removal of some factor (other than iron) upon which the reaction depends, showed that recrystallisation was not a satisfactory method for its removal.

Experiments were also carried out to determine whether the substance could be removed by adsorption methods. In preliminary experiments we found that, although substances such as cellulose or kieselguhr have no effect on the oxidation, the addition of kaolin to a solution of crystalline glutathione completely abolishes the oxygen uptake. This effect is, however, merely due to the removal of metals by adsorption on the kaolin, since the addition of

Fig. 3.

Fig. 4.

Fig. 3. Effect of recrystallisation. A, 3.5 mg. crystalline glutathione; B, 3.5 mg. recrystallised glutathione. In phosphate buffer at 20°. 0.0005 mg. iron added to B at arrow.

Fig. 4. Removal of iron by kaolin. A, 5 mg. crystalline glutathione; B, 5 mg. crystalline glutathione + 0.1 g. kieselguhr; C, 5 mg. crystalline glutathione + 0.1 g. filter paper; D, 5 mg. crystalline glutathione + 0.1 g. kaolin at 21°. 0.002 mg. Fe (as FeSO_4) added to D at first arrow. 0.1 g. more kaolin added at second arrow.

iron to the filtrate restores the rate of oxygen uptake to precisely its original value. From the experiment shown in Fig. 4, it can be seen that the reaction is catalysed by iron, that the added iron is removed by kaolin, and that kaolin inactivates the glutathione merely by adsorbing metals and does not adsorb the other factor, since the velocity returns to its original value on restoring iron to the system.

Treatment with kaolin therefore provides a very simple and convenient method for obtaining glutathione completely free from catalytic metals. All

that is necessary is to add kaolin to the solution, shake gently for a short time, and filter. Kaolin does not, however, inactivate either cysteine or the earlier glutathione preparations (see Fig. 5), for reasons which will be discussed later.

With adsorbents of this nature we were unable to remove the auxiliary catalytic factor, but with certain adsorbents of a more biological nature we were completely successful in doing so. As, however, these experiments are closely connected with those on the behaviour of the tripeptide towards tissue systems, they will be dealt with in the following section.

Fig. 5.

Fig. 5. Effect of kaolin on cysteine and on impure glutathione. A, 4 mg. old glutathione; B, 4 mg. old glutathione + 0.1 g. kaolin. In phosphate buffer at 40°. C, 5 mg. cysteine; D, 5 mg. cysteine + 0.1 g. kaolin. In phosphate buffer at 20°.

Fig. 6.

Fig. 6. Action of oxidised forms of old and new glutathione on thermostable tissue preparation. A, 4 mg. old oxidised glutathione + 0.2 g. muscle preparation; B, 4 mg. oxidised crystalline glutathione + 0.2 g. muscle preparation. In 3 cc. water at p_H 7.6 and 20°. In this and all other experiments the tissue was added to both flasks of the apparatus in order to eliminate the effect of any slight uptake by the tissue itself. The glutathione was of course added to the right-hand flask only.

Behaviour of glutathione towards tissue systems.

When the older preparations of glutathione, in either the reduced or the oxidised form, were added to thermostable muscle preparations at p_H 7.6 a large oxygen uptake resulted. In the case of the reduced form, the oxygen uptake was considerably greater than that given by the glutathione alone, owing to the simultaneous oxidation of protein. With the oxidised form, which of course gave no uptake in the absence of the tissue, the disulphide group was reduced by the —SH groups of the protein, and the oxygen uptake then followed as before.

Our first step in testing the behaviour of crystalline glutathione towards the thermostable system was to make certain that its oxidised form was capable of being reduced by the fixed —SH groups. For this purpose a solution of crystalline tripeptide in phosphate buffer was allowed to oxidise until it no longer gave a nitroprusside test. The resulting solution of oxidised glutathione was then added to a suspension of the muscle powder, allowed to stand for a short time, and then filtered. The filtrate gave a strong nitroprusside reaction, showing that pure oxidised glutathione, like the old, is readily reduced by the tissue powder.

In spite of this, however, the new glutathione shows a remarkable and complete difference in behaviour from the old; for on adding pure oxidised glutathione to the muscle powder not the slightest absorption of oxygen follows, although with the same muscle preparation the addition of the impure oxidised glutathione produces a large oxygen uptake. The results of a typical experiment are shown in Fig. 6.

It follows from this and similar experiments that pure glutathione is unable to bring about the oxidation of proteins. Moreover, since the tissue can reduce the glutathione it follows that reduced glutathione itself cannot oxidise in the presence of the muscle.

Yet more striking is the result when reduced crystalline glutathione is added to muscle powder. Not only is the tissue preparation itself not oxidised, but the oxidation of the glutathione is itself prevented by the presence of the tissue. The sulphhydryl group of the tripeptide is in fact quickly rendered stable to oxygen by the muscle powder. During the first few minutes a small oxygen uptake is sometimes observed, as the stabilisation is not quite instantaneous, but this ceases almost immediately, and the system can then be kept for long periods of time without undergoing oxidation. The total oxygen uptake in any case is never greater than a very small fraction of that needed to oxidise the sulphhydryl form to the disulphide. The experiment shown in Fig. 7 is typical of a large number of observations. For instance, out of 18 similar experiments with various preparations of tissue and of crystalline glutathione 14 showed rates of uptake (after the first few minutes) between 0 and 1 mm.³ per hour and the remaining 4 varied between 1 and 4 mm.³ per hour. The inactivation of the glutathione is therefore remarkably complete. The contrast between the behaviour of crystalline and impure glutathione is shown in Fig. 10.

After removal of the tissue by filtration the filtrate still shows no tendency to take up oxygen, as seen in Fig. 8. Nevertheless titration with iodine shows that it contains practically the whole of the reduced glutathione originally added; that is to say, only a small amount of the glutathione is adsorbed by the tissue.

200 mg. crystalline glutathione were dissolved in 60 cc. water and adjusted to p_H 7.6. 2 cc. of this were titrated with iodine (roughly $N/100$) using starch indicator. 2.57 cc. iodine were required. 2.5 g. thermostable muscle powder were then added to the solution, which was stirred for about an hour. After

Fig. 7.

Fig. 8.

Fig. 7. Stabilisation of glutathione by thermostable tissue preparation. *A*, 8 mg. crystalline glutathione; *B* and *C*, 8 mg. crystalline glutathione + 0.2 g. muscle preparation. In water at p_H 7.6 and 41°.

Fig. 8. Effect of impure glutathione on stabilised glutathione. *A*, 5 mg. crystalline glutathione; *B*, 5 mg. crystalline glutathione + 0.2 g. muscle preparation. In buffer at p_H 7.3 and 16°. At the first arrow the tissue was removed by filtration, and at the second arrow 3 mg. impure oxidised glutathione were added to *B*.

Fig. 9.

Fig. 10.

Fig. 9. Effect of iron on stabilised glutathione. *A*, 8 mg. crystalline glutathione; *B*, 8 mg. crystalline glutathione + 0.2 g. muscle preparation. In buffer at p_H 7.6 and 15°. At the first arrow the tissue was removed by filtration, at the second arrow 0.4 mg. Fe (as $FeSO_4$) was added, and at the third arrow 4 mg. impure oxidised glutathione.

Fig. 10. Comparative experiment with crystalline and impure glutathione in presence of tissue preparation. *A*, 9.6 mg. old (reduced) glutathione; *B*, 9.6 mg. old (reduced) glutathione + 0.2 g. muscle preparation; *C*, 5 mg. crystalline glutathione; *D*, 5 mg. crystalline glutathione + 0.2 g. muscle preparation. All in water at p_H 7.6 and 18°.

the tissue had been removed by centrifuging, 2 cc. of the solution required 2.33 cc. of the iodine. This tissue-treated solution, which of course gave a very strong nitroprusside reaction, was found in the Barcroft apparatus to give a quite negligible oxygen uptake.

Thus the addition of tissue powder to a solution of crystalline glutathione, followed after a short time by filtration, yields a solution of reduced glutathione which shows no tendency to undergo oxidation in air. We shall refer to glutathione which has been treated in this way as "stabilised glutathione."

We at first supposed that the stabilising effect of the muscle powder was similar to that of kaolin, and was due to the removal of metals by adsorption on the tissue. This is not the explanation, however, for whereas the addition of iron to the filtrate from the kaolin completely reactivated the glutathione, it had no effect upon the filtrate from the tissue, which remained quite stable to oxygen in the presence of even large amounts of iron or copper. Even such large amounts as 0.2 mg. of iron or copper have no effect. The effect of adding iron to stabilised glutathione is shown in Fig. 9, and precisely similar results were obtained with copper.

It is therefore quite clear that the tissue does not render the glutathione stable to oxygen by removing catalytic metals, but acts in some other way. Some evidence has already been given in the previous section that iron alone is not able to catalyse the autoxidation of glutathione; and this is convincingly shown by experiments of the type just described, for it is possible in this way to obtain strong solutions of reduced glutathione, containing large amounts of iron, which yet show no tendency to autoxidise. It is clear that iron and copper salts are themselves catalytically inactive in the reaction and do not form catalytic complexes with pure glutathione. As we suggested in the previous section, it appears that another factor must be present in addition to the iron in order that complexes capable of catalysing the oxidation of glutathione may be formed. If this is so the most probable explanation of the stabilising action of the muscle powder is that it removes the additional factor from the solution by adsorption. Glutathione can thus be rendered incapable of autoxidation in two ways: by the removal of metals by treatment with kaolin, or by removal of the other factor by treatment with muscle powder. In the first case the addition of iron to the filtrate reactivates it, since the second factor is not removed; in the second case this is lacking and the filtrate is therefore not reactivated by iron. It should, however, be reactivated by the addition of impure glutathione, which appears to contain the factor in considerable amount, as stated in the last section. The marked effect of adding glutathione from an earlier preparation to the stabilised glutathione is shown in Figs. 8 and 9.

The stabilised glutathione has lost not only its power to react with oxygen, but also its power of reducing methylene blue. (The reduction of methylene blue by cysteine was shown by Toda [1926] and by Harrison [1927] to be catalysed by metals, like the autoxidation.) The following experiment shows the effect of previous treatment with tissue upon the velocity of reduction.

A solution of crystalline glutathione in phosphate buffer, adjusted to p_H 7.6 and containing 4 mg. glutathione per cc., was prepared. This was divided into two portions: one portion was stabilised by treatment with muscle powder, which was then removed after about $\frac{3}{4}$ hour by filtration; the other portion was meanwhile kept in an evacuated Thunberg tube to prevent oxidation. Equal amounts of the two solutions, together with phosphate buffer, were then added to two Thunberg tubes so that each tube contained 3 mg. glutathione in 2 cc. liquid, then 0.3 cc. of 1/5000 methylene blue was added to each, and the reduction-times determined at room temperature in the usual way. An old preparation of glutathione under these conditions gave a reduction-time of about 40 minutes; the crystalline glutathione reduced in about 3 hours, while the stabilised glutathione did not reduce in 15 hours.

The behaviour of the —SH group of stabilised glutathione, which on our view is simply pure glutathione, resembles that of the fixed —SH groups of the thermostable preparations in being unable to react with either oxygen or methylene blue, even in presence of iron, and in becoming readily oxidised by both in the presence of impure glutathione.

Fig. 10 shows a comparative experiment with old and new glutathione preparations on the same sample of tissue. It confirms the results of Hopkins's experiments with the earlier preparations, and illustrates the marked difference in the action of the crystalline and the impure preparations. The reason for the failure of the tissue to stabilise the older preparations seems to be simply that they contain far too much of the auxiliary catalytic factor for the whole of it to be adsorbed by the tissue, so that the tissue becomes saturated with it before more than a fraction is removed from solution. Evidence for this will be given later.

Both cysteine and thioglycollic acid behave like the non-crystalline glutathione preparations, as Fig. 11 shows. This is to be expected, since the tissue does not remove the iron from the solution, and these substances are themselves capable of forming active complexes with iron. It is only those sulphhydryl compounds which are unable to form such complexes, and require the presence of another factor to activate the iron, which become stabilised.

Since it appears that cysteine can form such complexes and glutathione cannot, we thought it probable that other peptides of cysteine might also be incapable of forming active metal complexes, and either be stable to oxygen or become stabilised by tissue. On testing glycylcysteine¹ we found that it oxidised spontaneously at a slow rate, which was practically unaffected by the addition of iron, and that the addition of tissue powder had exactly the same stabilising effect as with crystalline glutathione. This experiment is shown in Fig. 12. This result seems to indicate that cysteine peptides generally may resemble glutathione in being unable to form complexes with metals which are able to catalyse their oxidation.

Chloroacetylcysteine was also tested, and found to behave like pure oxidised

¹ We are much indebted to Mr N. W. Pirie for giving us a sample of this peptide.

glutathione, that is to say, when it was added to tissue no oxygen uptake could be detected, although a nitroprusside test on the filtrate showed that reduction of the disulphide group by the tissue had taken place. The chloroacetyl-cysteine formed was therefore stable to oxygen in presence of tissue.

The action of haematin on stabilised and crystalline glutathione is very similar, for in both cases small amounts have no accelerating effect but large amounts (*e.g.* 0.5 mg.) definitely produce an acceleration. Fig. 13 shows the effect of adding a large amount of haematin to glutathione in the presence and absence of tissue preparation. While the inactivation is partially prevented by the presence of the haematin, the inhibitory effect of the tissue is still very marked.

Fig. 11.

Fig. 12.

Fig. 11. Action of cysteine and thioglycolic acid on tissue preparation. *A*, 2 mg. cysteine + 0.001 mg. Fe; *B*, 2 mg. cysteine + 0.001 mg. Fe + 0.2 g. muscle preparation; *C*, 3 mg. thioglycolic acid; *D*, 3 mg. thioglycolic acid + 0.2 g. muscle preparation. All in water at p_H 7.6 and 16°. The oxygen-equivalents of the cysteine and thioglycolic acid respectively are indicated by the vertical lines on the right of the figure.

Fig. 12. Stabilisation of glycylcysteine. *A*, 5 mg. glycylcysteine; *B*, 5 mg. glycylcysteine + 0.2 g. muscle preparation. In phosphate buffer at p_H 7.6. At the arrow 0.001 mg. Fe was added.

In further experiments on the stabilisation of glutathione by tissue preparations it was found that the p_H of the solution had a considerable influence, for while at p_H 7.6 the stabilisation was usually complete no stabilisation at all could be detected at p_H 9.0. The presence of the tissue had in fact no effect on the velocity of oxidation in alkaline solution. A solution of glutathione which had been stabilised at p_H 7.6 did not become reactivated by making it alkaline, so that it appears that the tissue does not adsorb the catalytic impurity in alkaline solutions. The significance of this result will appear later.

The thermostable tissue preparation consists mainly of insoluble protein material, and we therefore investigated the action of other proteins upon the rate of oxidation of aqueous glutathione solutions, in order to see whether they showed the same stabilising effect as the tissue powder. It was found that gelatin, caseinogen and egg-albumin did not affect the rate of oxygen uptake, but that coagulated egg-albumin slowed it considerably. At first it appeared possible that the presence of a sulphhydryl group in the protein molecule might be essential for it to exert a stabilising action, for the tissue preparations and coagulated egg-albumin possess sulphhydryl groups, whereas gelatin, caseinogen and native egg-albumin do not. This was, however, shown not to be the case by the fact that denatured globin¹, which shows no —SH reaction, stabilised the glutathione almost completely. It appears that the heterogeneity of the system is the determining factor. Some of these experiments are shown in Fig. 14.

Fig. 13.

Fig. 14.

Fig. 13. Effect of larger amounts of haematin. *A*, 5 mg. crystalline glutathione; *B*, 5 mg. crystalline glutathione + 0.5 mg. haematin; *C*, 5 mg. crystalline glutathione + 0.2 g. muscle preparation; *D*, 5 mg. crystalline glutathione + 0.5 mg. haematin + 0.2 g. muscle preparation. All in buffer at p_H 7.6 and 20°. The tissue used in this experiment had been freed from haematin by extraction with pyridine, followed by ether.

Fig. 14. Effect of proteins on autoxidation of glutathione. *A*, 5 mg. crystalline glutathione; *B*, 5 mg. crystalline glutathione + 0.04 g. gelatin; *C*, 5 mg. crystalline glutathione + 0.2 g. egg-albumin; *D*, 5 mg. crystalline glutathione + 0.2 g. denatured egg-albumin; *E*, 5 mg. crystalline glutathione + 0.07 g. denatured globin. All in phosphate buffer at p_H 7.6 and 16°.

From the biological point of view it was obviously important to determine whether fresh unheated tissues could also stabilise glutathione, or whether this property was restricted to tissue preparations which had been heated. It was possible that the power to remove the catalytic factor by adsorption had been conferred upon the thermostable preparations by the heat-coagulation of their proteins. In order to test this, fresh rat muscle was minced, well washed with

¹ We are indebted to Mr R. Hill for samples of globin prepared from horse and sheep blood.

tap water, and added to a solution of crystalline glutathione. The results of one experiment are shown in Fig. 15. In another experiment with a different rat a larger inhibition of oxygen uptake was obtained. The fact that contact with fresh tissues tends to render glutathione stable to oxygen cannot but be of considerable importance in connection with the mode of action of glutathione *in vivo*.

Oxidation of unsaturated fatty acids.

So far we have been dealing with the experiments on the oxidation of protein, in which the oxidation of the sulphydryl group of glutathione is involved. Hopkins also found that glutathione could catalyse the oxidation of unsaturated fats and fatty acids, particularly in acid solution. In this catalysis the oxidation of the $-SH$ group is not involved, and there was therefore no reason to anticipate that the phenomena connected with stabilisation should affect this action. In fact the behaviour of the crystalline glutathione towards fats appears to be exactly the same as the non-crystalline. In Fig. 16 is shown a comparative experiment on linoleic acid at p_H 3.5 with old and new glutathione. It will be seen that the induced oxygen uptake is the same in both cases.

Fig. 15.

Fig. 16.

Fig. 15. Inhibition of autoxidation of glutathione by washed rat muscle. *A*, 5 mg. crystalline glutathione + 0.001 mg. Fe; *B*, 5 mg. crystalline glutathione + 0.001 mg. Fe + 1 g. washed rat muscle. In phosphate buffer at p_H 7.6 and 16°. The muscle and iron were added to both flasks of the apparatus, so as to compensate for any residual respiration.

Fig. 16. Oxidation of linoleic acid by old and new glutathione preparations. *A*, 8 mg. crystalline glutathione; *B*, 8 mg. impure glutathione; *C*, 10 mg. linoleic acid + 8 mg. crystalline glutathione; *D*, 10 mg. linoleic acid + 8 mg. impure glutathione. All in water at p_H 3.5 and 39°. The linoleic acid was present as a fine emulsion, and it was added to both flasks of each apparatus *C* and *D*, in order to compensate for its spontaneous autoxidation.

Several samples of linseed oil were also tested at p_H 7.6, but with the samples available we obtained no oxidation either with pure or impure glutathione or with cysteine, thus confirming the results of Allott [1927].

We have not observed any differences in behaviour between the old and new glutathione preparations as far as the oxidation of fats is concerned.

Nature of the catalytic impurity. Reactivation of stabilised glutathione.

Since the catalytic substance is present in crystalline glutathione only in extremely small amounts (quite insufficient to affect the analytical figures) it is not practicable to isolate it and identify it directly. Evidence as to its nature must therefore mainly rest upon the comparison of its behaviour with that of known substances. While we cannot claim to have definitely identified the substance, we have obtained evidence which gives distinct indications of its nature; and it appears very probable that it is either free cysteine or some very closely related substance. Its properties correspond almost exactly with those of free cysteine; nevertheless certain quantitative experiments seem to show that its activity is somewhat greater than that of cysteine itself.

Cysteine is known to be capable of forming active complexes with iron and copper, it is known to be present in considerable amounts in the older glutathione preparations, it is the most likely impurity to occur in the new glutathione preparations (since it is precipitated by the Hopkins copper method), and it would probably also occur in traces in preparations of other cysteine peptides.

On this hypothesis then the catalyst which brings about the oxidation of glutathione is a cysteine-iron (or copper) complex. According to the theory of cysteine autoxidation recently put forward by Michaelis [1929] the oxidation of cysteine is due to the catalytic action of a cysteine-iron complex rather than of iron itself. In that case both glutathione and cysteine would be oxidised by the same catalyst. This would explain a fact which has hitherto been somewhat difficult to account for; namely, that the p_H -oxidation-velocity curves for cysteine and (impure) glutathione (in presence of a trace of copper) have the same form, with a marked optimum at p_H 7.5, although the mode of ionisation of the two substances is different. Other sulphydryl compounds, such as thio-glycollic acid, show quite different curves, with no optimum in this region. This would seem to indicate that the catalyst is the same in both cases.

We were first led to think that the extra catalytic factor might be cysteine by the following circumstance. In preparing glutathione by Hopkins's [1929] method, if the final solution, obtained by the decomposition of the crystalline copper compound by means of H_2S , is quickly concentrated *in vacuo* and allowed to crystallise after adding alcohol in the usual way, the whole of the glutathione is obtained as a non-deliquescent crystalline preparation, with the properties described in this paper. If, however, the solution is allowed to stand for a few hours, or the evaporation carried out slowly, the preparation obtained is only partially crystalline and resembles the older preparations in being deliquescent. Slight hydrolysis of the tripeptide appears to have taken place with the production of a small amount of free cysteine; as shown by the fact that the Sullivan [1926] test, which is negligibly weak with crystalline glutathione,

became definitely positive with this preparation. The products of hydrolysis apparently interfere with the crystallisation of the tripeptide. This preparation, which we shall refer to as "deliquescent glutathione," and which we may repeat is obtained from the crystalline copper compound of the tripeptide, behaves with tissue powder quite differently from the crystalline preparations, but in very much the same way as the older preparations, as shown in Fig. 17. It appeared therefore that glutathione containing a sufficient amount of free cysteine to give a positive Sullivan reaction, as in the case of the older and the deliquescent preparations, was not stabilised by the tissue; whereas preparations which gave a negative Sullivan reaction were stabilised. That is to say, where the glutathione contained more than a minute trace of cysteine the active factor was present in too large an amount to be removed by adsorption on the tissue.

Fig. 17.

Fig. 18.

Fig. 17. Action of tissue preparation on crystalline and "deliquescent" glutathione. A, 7 mg. crystalline glutathione; B, 7 mg. crystalline glutathione + 0.2 g. muscle preparation; C, 8 mg. "deliquescent" glutathione; D, 8 mg. "deliquescent" glutathione + 0.2 g. muscle preparation. In phosphate buffer at p_H 7.6 and 20° . 0.01 mg. Fe (as $FeSO_4$) added to B at arrow.

Fig. 18. Action of "reactivated" glutathione on thermostable tissue preparation. A, 10 mg. "reactivated" glutathione; B, 10 mg. "reactivated" glutathione + 0.2 g. muscle preparation. In water at p_H 7.5 and 40° .

This experiment suggested that crystalline glutathione which had been allowed to stand in aqueous solution for some time might behave similarly. A solution containing 100 mg. crystalline glutathione in 10 cc. water was accordingly allowed to stand (unneutralised) for 12 hours at 40° , after which it was adjusted to p_H 7.5. It was then found that the solution gave a definitely positive Sullivan reaction, indicating that a small amount of hydrolysis had taken place. The oxygen uptake of this solution was accelerated by adding a

trace of iron, like that of the impure glutathione, and it also behaved like the latter with tissue: that is to say its oxidation was not inhibited by the tissue, and it was able to catalyse the oxidation of the protein, as shown in Fig. 18. This experiment has been repeated with other crystalline preparations with similar results.

These experiments show clearly that the catalytic factor can be formed from the pure tripeptide itself, and that its appearance is accompanied by the liberation of a small amount of free cysteine.

We attempted to make use of the delicate and specific Sullivan test for free cysteine for the purpose of estimating colorimetrically the amounts present in the different preparations of glutathione, but we found that in presence of glutathione the development of the colour is markedly inhibited. For instance 1 mg. of cysteine gave a strong Sullivan reaction, but 1 mg. of cysteine plus 9 mg. of glutathione gave only a weak colour. For this reason it is difficult to use the Sullivan method for the estimation of small amounts of cysteine in glutathione preparations. It can, however, be said that there are indications that the strength of the reaction runs roughly parallel with the amount of catalytic factor present. All the older glutathione preparations tested gave a strong (though rather variable) test, the deliquescent preparations and such solutions as that used in Fig. 18 gave positive, though weaker, tests, while crystalline glutathione gave only a negligible colour. Attempts to obtain quantitative results by using a series of standards containing varying amounts of cysteine and glutathione were not very successful, but appeared to show that between 1 and 5 % of the total sulphydryl in the solutions used in Fig. 18 was present as free cysteine.

We may mention here that the addition of ferric chloride to the impure glutathione produces the transient blue colour characteristic of cysteine, while with crystalline glutathione or with glycylcysteine no blue colour is produced, and only a pale olive tint remains.

In order that the hypothesis that the catalytic substance is cysteine may be tenable, the following points must be verified. (1) A mixture of crystalline glutathione with a small proportion of cysteine must behave like the non-crystalline glutathione preparations. (2) The thermostable muscle powder must be capable of removing small amounts of cysteine from solution by adsorption. (3) The addition of cysteine to stabilised glutathione must reactivate it.

All these points have been verified by experiment.

Fig. 19 shows that the addition of cysteine to crystalline glutathione prevents the latter from being inactivated by the tissue; and the large effect of adding small quantities of cysteine to reduced or oxidised glutathione is illustrated in Fig. 20.

The presence of phosphate tends to prevent the oxidation of protein [see Hopkins, 1925], but Fig. 21, which gives an experiment carried out in the absence of phosphate, shows that extended oxygen uptakes with oxidation of

protein can be obtained on adding a trace of cysteine to the crystalline glutathione.

With regard to the second point, when very small amounts (less than 0.1 mg.) of cysteine are added to 0.2 g. of muscle powder at p_H 7.6 no oxygen uptake is produced. On filtering off the tissue, the liquid is found to give no

Fig. 19. Prevention of stabilisation by presence of cysteine. *A*, 5 mg. crystalline glutathione; *B*, 5 mg. crystalline glutathione + 0.2 g. muscle preparation; *C*, 5 mg. crystalline glutathione + 1 mg. cysteine; *D*, 5 mg. crystalline glutathione + 0.2 g. muscle preparation + 1 mg. cysteine. In phosphate buffer at p_H 7.6 and 20°. The cysteine and muscle were added to both flasks of the apparatus. The uptake of the cysteine itself is therefore eliminated from the curves.

Fig. 20. Effect of addition of a trace of cysteine to pure reduced and oxidised glutathione. *A*, 0.1 mg. cysteine + 0.3 g. muscle preparation; *B*, 5 mg. crystalline glutathione + 0.1 mg. cysteine + 0.3 g. muscle preparation; *C*, 5 mg. oxidised crystalline glutathione + 0.2 g. muscle preparation; *D*, 0.1 mg. cysteine + 0.2 g. muscle preparation; *E*, 5 mg. oxidised crystalline glutathione + 0.1 mg. cysteine + 0.2 g. muscle preparation. In phosphate buffer at p_H 7.6 and 40°. The oxygen equivalent of the cysteine added is indicated by the vertical line on the right of the figure.

nitroprusside reaction, even after adding cyanide to reduce any cysteine which may have been formed. The cystine has in fact been completely removed from the solution by adsorption on the tissue. This does not occur, however, in alkaline solutions: at p_H 9 the tissue does not adsorb the cysteine. Thus

under conditions where the muscle powder adsorbs cysteine it is capable of stabilising glutathione, and under other conditions, where it cannot stabilise glutathione, it does not adsorb cysteine.

Moreover the muscle powder may be rendered incapable of adsorbing cysteine by previously exposing it to excess of cysteine so as to saturate it, and then removing the free cysteine by very thorough washing with water. After this treatment the tissue was incapable of stabilising glutathione.

Fig. 21. Oxidation of protein by crystalline glutathione in presence of small amounts of cysteine. *A*, 5 mg. crystalline glutathione; *B*, 5 mg. crystalline glutathione + 0.2 g. muscle preparation; *C*, 5 mg. crystalline glutathione + 0.2 g. muscle preparation + 0.25 mg. cysteine; *D*, 5 mg. crystalline glutathione + 0.2 g. muscle preparation + 1 mg. cysteine. In water at p_H 7.6 and 18°. The cysteine was added to both flasks in *C* and *D*. The vertical lines indicate the oxygen equivalent of the amounts of cysteine added.

Another point of interest is that in the presence of excess of glutathione the tissue seems to have much less power of adsorbing cysteine. This is possibly because it can adsorb both compounds so that in presence of an excess of glutathione the surface would be mainly saturated with this and little cysteine would be adsorbed. Fig. 22 illustrates this point. Neither the cysteine nor the glutathione separately gave any oxygen uptake in presence of the tissue: the cysteine because it was present in sufficiently small quantity to be completely adsorbed by the tissue, the glutathione because it became stabilised by the tissue in the usual way. When both were present together, however, a considerable oxygen uptake was produced.

With regard to the third point, the addition of a trace of cysteine to stabilised glutathione (after the tissue has been filtered off) restores the power to take up oxygen. The effect of adding different amounts of cysteine is shown in Fig. 23.

All the evidence given hitherto in this section seems to indicate that the impurity necessary for the oxidation of glutathione is cysteine. The fact that kaolin removes the catalytic iron from glutathione preparations but not from cysteine might be thought to be evidence to the contrary, since this view supposes that the active iron is present in both cases as a complex with cysteine.

But the difference is easily understood if we assume that the complex is partly dissociated into free iron and cysteine, and that the kaolin only adsorbs free iron. For in accordance with the principle of mass action the amount of free iron will decrease as the cysteine concentration increases; so that in the glutathione solutions, where the cysteine is present only in traces, the kaolin may be able to remove practically the whole of the iron, while in the cysteine solutions the concentration of free iron will be negligible, and therefore very little will be adsorbed.

Fig. 22.

Fig. 23.

Fig. 22. Effect of very small amounts of cysteine. *A*, 5 mg. crystalline glutathione + 0.2 g. muscle preparation; *B*, 0.05 mg. cysteine + 0.2 g. muscle preparation; *C*, 5 mg. crystalline glutathione + 0.2 g. muscle preparation + 0.05 mg. cysteine. In water at p_H 7.6 and 40° . The oxygen-equivalent of the cysteine added was about 2 mm.³. It was found that in the absence of the glutathione the tissue completely adsorbed the cysteine.

Fig. 23. Effect of addition of cysteine to stabilised glutathione. A solution of stabilised glutathione was prepared by treatment with thermostable preparation, followed by filtration. Various amounts of cysteine were then added to the filtrate as follows: *A*, 2 mg. stabilised glutathione + 0.001 mg. Fe; *B*, 2 mg. stabilised glutathione + 0.001 mg. Fe + 0.1 mg. cysteine; *C*, 2 mg. stabilised glutathione + 0.001 mg. Fe + 0.2 mg. cysteine. In phosphate buffer at p_H 7.6 and 20° . The cysteine and iron were added to both flasks of the apparatus.

Quantitative experiments seem to show that while cysteine (together with iron) can act as catalyst in the oxidation of glutathione, its activity is somewhat less than that of the active substance present in solutions of the crystalline, "deliquescent" and "reactivated" glutathiones. The results given in Fig. 23 show that the presence of 5 % of free cysteine in the glutathione would account for an oxygen uptake of about 50 mm.³ per hour for 5 mg. of glutathione. Now the rate of oxidation of crystalline glutathione under similar conditions is about 30 mm.³ per hour per 5 mg. If this oxidation were due to the presence of cysteine, crystalline glutathione must contain 3 % of free cysteine. This, however, does not seem possible. The limits of error of the analytical figures would only permit of the presence of 0.5 % of free cysteine at most (assuming,

however, that cysteine is the only impurity present), and an even smaller amount than 0.5 % could have been detected by the Sullivan reaction in spite of the inhibitory effect of the glutathione. Similar reasoning shows that the "deliquescent" glutathione used in the experiment of Fig. 17 would have to contain about 8 % of free cysteine, whereas the Sullivan reaction indicated that not more than about 1 % was present. Added cysteine therefore hardly seems to be sufficiently active.

Nevertheless in view of the somewhat complicated nature of the reactions involved we do not feel justified in claiming that these considerations are conclusive.

DISCUSSION.

We consider that the experiments given in this paper show that pure glutathione is not appreciably autoxidisable, even in presence of iron.

There are four possible interpretations of the effect of the muscle powder in preventing the oxidation of glutathione. (1) The tissue may remove the necessary iron by adsorption. (2) Some substance which inhibits the catalytic action of the iron may pass into the solution from the tissue. (3) The glutathione may be converted into a non-autoxidisable isomeric modification, the tissue acting as a catalyst in the process. (4) The tissue may remove from the solution some impurity other than iron, which co-operates with the iron in catalysing the oxidation.

The first possibility is disposed of by the fact that the addition of iron after filtering off the tissue powder does not render glutathione autoxidisable.

The second is made improbable in view of the very thorough extraction undergone by the tissue preparation. The tissue is first very thoroughly extracted with water, then with boiling water, then with absolute alcohol for several hours in a Soxhlet apparatus, and finally with ether. In some cases it was also extracted with pyridine and again with ether. After such treatment it is difficult to believe that any soluble material is left.

This interpretation seems also to be excluded by the form of the curves. The stabilisation is not instantaneous, but takes a certain time to become established. The initial velocity of oxidation in presence of tissue is not much below that in its absence, but it quickly falls off to a negligible value. On the second interpretation this period would correspond to the time taken for the inhibitor to diffuse out of the tissue. If therefore the tissue is allowed to remain in the buffer solution for say 2 hours before adding the glutathione, the inhibitor should have had time to diffuse into the solution, and the stabilisation should now be instantaneous. Actually, however, this procedure has no effect on the length of time taken to attain complete stabilisation.

The hypothesis is finally disposed of by the following consideration. In a series of experiments with different amounts of tissue it was found that 0.08 g. of the tissue was required in order to stabilise 5 mg. of glutathione. Smaller amounts of tissue produced only a partial inhibition. On the hypo-

thesis that the tissue was supplying an inhibitor this would mean that that amount of tissue contained just enough of the inhibitor to inactivate the metals present. The amount of tissue used in most of our experiments, 0.2 g., would therefore only be able to inactivate about twice as much iron as was originally present in the solution. Yet after removing the tissue by filtration, many (*e.g.* 1000) times this amount of iron can be added without re-establishing any uptake of oxygen. The second interpretation may therefore be neglected.

In order to explain the stabilisation of glutathione by its conversion into an isomeric modification it would seem to be necessary to postulate some change involving the sulphhydryl group, so that stabilised glutathione would not contain a free sulphhydryl group. Any change not involving the sulphhydryl group would by itself hardly be expected to prevent the autoxidation. Yet stabilised glutathione does not appear to have undergone any change in its molecule. It titrates normally with iodine, it is precipitated normally by the copper method, and it gives with nitroprusside a sulphhydryl reaction of undiminished intensity. It is true that in performing the nitroprusside test the solution is made alkaline with ammonia, which might produce a tautomeric change; but we found that this treatment did not reactivate stabilised glutathione in the least. Moreover on this view the tissue powder would be acting as a catalyst for the change, and the rate of stabilisation should vary with the amount of tissue present. Actually the time taken to reach stabilisation is independent of the amount of tissue within wide limits. And finally this hypothesis provides no explanation of the reactivation of the stabilised glutathione by the addition of a little impure glutathione or cysteine.

According to the remaining hypothesis the tissue removes some substance which, in co-operation with iron, is responsible for the catalysis. It has been clearly shown that iron alone cannot catalyse the oxidation of the cysteine peptides, neither can it form active complexes with them. Nevertheless that the oxidation depends upon the action of iron is shown by the experiments with kaolin. We are therefore compelled to assume that the active catalyst is a complex of iron with some other impurity, in the absence of which glutathione would be stable to oxygen, even in presence of iron. Several lines of evidence have been shown in this paper to converge in support of this view, and it appears to provide an adequate explanation of the facts.

The fact that iron and copper can form active complexes with cysteine but not with the cysteine peptides is of importance in connection with theories of oxidation-catalysis by iron. It appears that the amino-group of cysteine must be left free in order that active complexes with iron may be formed.

With regard to the nature of the impurity in crystalline glutathione which forms active complexes with iron, we can only say that its behaviour is similar to that of free cysteine. The identity of the impurity in these preparations is perhaps not a matter of very great consequence. In any case cysteine is capable of acting in such a way, and there can be little doubt that in the work done on the autoxidation of the older glutathione preparations (as for instance that of

Dixon and Tunnicliffe [1923], Harrison [1924], Dixon [1928]) the effects were due to the presence of cysteine, as also in the case of the oxidations of proteins studied by Hopkins [1925].

We do not picture the cysteine impurity as acting by the alternate oxidation and reduction of its sulphur group, but merely as converting the iron into a catalytically active complex. It is the sulphur group of glutathione which undergoes alternate oxidation and reduction in the presence of tissue, the ferrocysteine catalysing the reaction between glutathione and oxygen.

The question now arises as to how glutathione is oxidised in the tissues. The following possibilities must be considered. (1) Autoxidation catalysed by ferrocysteine or similar complexes. (2) Autoxidation catalysed by haematin. (3) Oxidation by means of the cytochrome system. (4) Oxidation by hydrogen peroxide, either directly, or indirectly as suggested by Szent-Györgyi [1928].

The first method seems to be the most probable. It does not seem to be definitely known at present whether the tissues contain free cysteine in appreciable quantities or not, but it seems quite probable that sufficient amounts would be present to account for the oxidation.

We have shown that small amounts of haematin do not affect the autoxidation, and it seems questionable whether sufficient haematin is present in the cells to catalyse the reaction.

Keilin [1930] has shown that cysteine is very rapidly oxidised by the cytochrome—indophenol oxidase system, with uptake of oxygen, and it might be expected that glutathione would be oxidised by the same system. Dr Keilin has kindly allowed us to state, however, that he has found that crystalline glutathione is not oxidised by this system, and that it reduces cytochrome only with extreme slowness (cf. our results on haematin given previously).

Further work is at present being carried out on the possibility of oxidation by hydrogen peroxide.

With the data at present available no final statement can be made with regard to the mode of oxidation of glutathione in the tissues.

SUMMARY.

1. Previous work on oxidation-catalysis by glutathione, in which impure preparations were used, has been repeated with crystalline glutathione. Some significant differences in behaviour have been found.

2. Solutions of crystalline glutathione absorb oxygen at the comparatively slow rate of about 30 mm.³ per hour per 5 mg. glutathione (at p_H 7.6 and 20°).

3. As in the case of cysteine, this oxidation is due to the presence of traces of catalytic metals. These can be removed by adsorption on kaolin. The resulting solutions do not absorb oxygen, but are reactivated by the addition of a trace of iron, which brings back the autoxidation to its original rate. Cysteine is not inactivated by kaolin.

4. The autoxidation of solutions of crystalline glutathione, unlike that of cysteine, is not accelerated by the addition of iron.

5. Evidence has been obtained that the autoxidation of glutathione depends on the co-operation of two factors, present in traces as impurities in the glutathione preparations; namely, iron (or copper), and some substance able to form catalytically active complexes with metals. With crystalline glutathione the rate of oxidation is limited by the amount of the second factor present, and not by the iron.

6. Crystalline glutathione, unlike the earlier preparations, produces no oxygen uptake when added to "thermostable tissue preparations." When the disulphide form is added it becomes reduced by the tissue, but no oxygen uptake results. When the sulphydryl form is added not only does it fail to oxidise the protein, but its own autoxidation is completely inhibited.

7. This inhibition is not due to the removal of catalytic metals, as with kaolin; for, on filtering off the tissue, solutions of reduced glutathione are obtained which are unable to react with oxygen (or methylene blue) even after the addition of iron or copper. They are, however, reactivated by the addition of a small amount of impure glutathione.

8. It is probable that the tissue inactivates the glutathione by removing the impurity which forms active complexes with metals. Free iron and copper are catalytically inactive in the oxidation of glutathione, and glutathione is unable to form active complexes with them. It follows that pure reduced glutathione is not autoxidisable, even in presence of iron or copper.

9. Other peptides of cysteine behave in a similar way to glutathione.

10. Certain other proteins behave like the "thermostable preparations" in inactivating glutathione. The oxidation of glutathione is also inhibited by fresh washed muscle.

11. Evidence is given that the catalytic substance, other than metals, concerned in the oxidation of glutathione can be formed, under certain conditions, from the glutathione molecule itself.

12. A mixture of crystalline glutathione and free cysteine behaves in the same way as the earlier glutathione preparations.

13. While the properties of the catalytic impurity in the glutathione preparations can be very closely imitated by free cysteine, quantitative experiments do not altogether support the view that this substance is cysteine itself.

14. In the oxidation of unsaturated fatty acids crystalline glutathione shows the same catalytic power as the earlier preparations.

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